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Development of Tupaia javanica Immortal Hepatocytes Culture for Javan Gibbon Hepatitis B Virus Replication

Maryati Surya[a*], Diah Iskandriati[b], Dondin Sajuthi[c], Joko Pamungkas[d]

[a,b,c,d]*Primate Research Center, Bogor Agricultural University, JalanLodaya II no 5 Bogor, 16151, Indonesia*

[a]*Email: msurya1506@yahoo.com*

Abstract

Hepatitis B is a liver inflammation caused by hepatitis B virus (HBV). Hepatocytes culture is an *in vitro* model widely used to study various physiological and pathological aspects of HBV. Referring to 3R principles (*Reduction, Refinement, Replacement*), it is important to minimize the use of animals in research (in this study :*Tupaia*). Therefore, in this research, we developed the immortal *Tupaia* hepatocytes culture that suitable with Javan gibbon HBV replication. It is hoped that the research results can be further used by other researchers in developing hepatitis B medicines or vaccines. The study showed hepatocytes primary culture that has been transformed shows the existence of telomerase activity through telomerase test on day 8 (0.000159 amol), day 22 (0.000266 amol), day 29 (0.000762 amol), day 50(0.00502 amol) and day 64 (0.000076 amol) after transfected. This culture is able to replicate Javan gibbon HBV to the eighth day following inoculation.

Keywords: Immortal hepatocytess culture; Tupaia javanica; Javan gibbon HBV.

1.Introduction

Hepatitis is an inflammation of the liver which may be caused by a virus, bacteria, drugs or alcohol use. In most cases, the cause is a hepatitis virus. To date, there are five main hepatitis viruses, namely type A, B, C, D, and E. The lastly mentioned four viruses attract most attention because of their lethality and epidemic potential.

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* Corresponding author.

Specifically, type B and C can lead to chronic liver infection to hundred million people that may go on to develop cirrhosis and cancer. Worldwide, it is estimated that two million people are infected by hepatitis B virus (HBV), and over 350 million others have chronic (long term) liver infection [1, 2].

The use of either *in vivo* or *in vitro* model is one of the aspects of hepatitis B research. *In vivo* ideal model for HBV infection must be able to represent HBV infection that happens in human, for example how a virus interacts with human hepatocytes and the effect of human immunity system on HBV infection. The primary obstacle to conduct hepatitis B research is the lack of good *in vitro* as well as *in vivo* systems using small animal model, which can be infected with HBV and its replication [3]. Generally, animal model can only represent the criteria partially, and for the last 10 years, the ideal animal model in HBV research was chimpanzees (*Pan troglodytes*). Since chimpanzees are categorized as endangered primate, then their utilization is limited, additionally the cost of handling is high and they are difficult to handle due to their size; therefore, a substitute animal model is needed.

Research showed that several small animal species can be used as animal model of HBV. *Tupaia belangeri* (treeshrew) which is native to China [4] is one of the small species frequently used in research related to hepatitis B. Those animals could be infected by HBV both *in vivo* and *in vitro* and were able to develop acute and chronic hepatitis [5-8]. The difficulties to bring these animals out from their habitat into Indonesia becomes a limitation on hepatitis B research development in this country. Indonesia is a country with mega diversity whose flora and fauna are innumerable. One of the fauna in Indonesia is a treeshrew (e.g. *T. javanica* dan *T. glis*) and there has not been any biomedical researches that use these species. Therefore, further exploration needs to be done to see the capability of hepatocytes from Indonesian *Tupaia* to support the replication of HBV as has been done in *T. belangeri*.

Tupaia javanica is one of Indonesian *Tupaia* species that inhabits Sumatera, Jawa, Bali and Kalimantan [9]. To date, there are no biomedical researches recorded using this species. This can be seen as an opportunity to utilize Indonesian fauna wealth for human welfare; hence, the capability of Indonesian *Tupaia sp.* hepatocytes to support the replication of HBV needs to be further explored as has been done in *T. belangeri* hepatocytes. Primate Research Center, Bogor Agricultural University (IPB PRC) Indonesia was succeeded to developed primary *T. javanica* hepatocytes culture and succeeded to infect it with Javan gibbon (*Hylobates moloch*) HBV(GiHBV) (unpublished).

Regarding to 3R principles (Reduction, Refinement, Replacement) that adhere to research with animal model, it is important to note the application of Reduction and Replacement principles which is to minimize the use of animal (in this study : *Tupaia*) in constructing primary hepatocytes culture. Consequently, a system to substitute the primary culture needs to be developed, i.e. immortal hepatocytes culture.

In this study, an immortal hepatocytes culture from *T. javanica* native to Indonesia will be developed. This culture was innoculated with GiHBV to study the replication capability of the virus in *T. javanica* immortal hepatocytes culture.

2. Materials and Methods

2.1 Isolation and primary Tupaia hepatocytes (PTH) culture

Three wild adult *T. Javanica* Horsfield 1822 (identified by the Indonesian Institute of Science Number 269/IPH.I.03/KS.02/IX/2013 dated 26 September 2013, Figure 1) were maintained in the quarantine facility of Primate Research Center, Bogor Agricultural University (IPB PRC). All animals procedures were performed by veterinarians at the IPB PRC, and animals protocols have been evaluated and approved by IPB PRC Institutional Animal Care and Use Committee (IACUC) Number PRC-IPB-13-D004 dated 30 April 2013. Procedure of hepatocytes isolation was refered to previous study [10] with the following modification: pre-perfusion washing solution (Hanks Balanced Salt Solution dari Invitrogen, USA; cat# 14170-112, 5 mM EGTA, 0,25 ug/mL amphotericin B), and perfusion solution (DMEM, 100x CaCl2, 1% colagenase II) was used to remove red blood cells from the liver. This step was done using 50 ml syringe until the liver was semi-solid and pale. Livers then were minced and incubated at 37° C for 5-10 minutes, centrifugated at 300-500 g for 5 minutes, and washed using saline buffer to obtain cell pellets. Cells were plated in 24-well culture plate at 10^4 cells per wellusing growth media (HBM and HCM hepatocytes*s* media from Lonza USA supplemented with 20% Fetal Bovine Serum)at 37° C and 5% CO_2.

2.2 Transformation to immortal hepatocytes culture

The primary hepatocytes culture that has reached 80% confluence was then given a mixture of Lenti-SV40 solution from Capital Biosciences (cat# CIP-0011) and growth media, then incubated overnight at 37°C, 5% CO2, as described by the manufacture's manual. The transformation media was removed and replaced by 1 ml complete growth media. The hepatocytes population was subcultured as many as 7 passages in the effort to establish immortal hepatocytes culture; and to assess the transformation,telomerase test was carried out using Quantitative Telomerase Detection Kit (Allied Biotech, Inc, USA).

2.3 Virus inoculation on to the cells

GiHBV isolate was complimentary obtained from the virus collection of IPB PRC Indonesia. HBsAg titer (IU/mL) of the isolates was 60,000 IU/mL (GiHBV). Virus suspension was added to PTH on day 50 after transfection followed by 18 hours of incubation at 37°C with 5% CO_2. The cells were washed with saline buffer to remove the excess viruses and then later growth media were added. The released viruses were collected from infected cells media on days 1 to 8.

2.4 Detection and quantification of HBV replication

Detection of released HBV in the media was performed using Polymerase Chain Reaction (PCR) and the number of viral DNA was measured using quantification PCR (qPCR). The primer set used in this method was described previously [11]. This primer set amplified the partial S gene of HBV andyielded aproximately 456 bp fragment of this gene. DNA was extracted using QiAmp Blood DNA Mini Kit (Qiagen, USA) according the manufature's procedures.

PCR amplification conducted using conventional PCR (Applied BioSystem 9700, USA) at the following conditions: 94°C for 10 min, followed by 40 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, with a final extension at 72°C for 10 min. PCR reaction at 25 µl total reaction containing 10 pMol/ml of each primer, 12.5 ul Kappa HotStart ReadyMix (KAPA Biosystems, USA), 5 µl DNA and adjusted with nuclease free water to 25 µl. Amplicons (5 µl) were visualized on 1% agarose gel and sized against a 100-bp DNA ladder (Invitrogen, USA).

For HBV replication quantification, we used iQ5 Real Time PCR (BIO-RAD, USA). Real time PCR amplification was performed by the following protocol: denaturation for 2 min at 98°C, amplification for 40 cycles consisting of 5 sec at 98°C and 10 sec at 55°C. The reactions were 10 pMol/ml of each primer, 12.5 µl SsoFast EvaGreen Supermix (BIO-RAD, USA), 5 µl DNA and adjusted with nuclease free water to 20 µl.

3. Results and Discussion

The liver organ was colected from an adult male *Tupaia javanica* Horsfield, 1822 (figure 1) by a veterinarian of IPB PRC Indonesia in compliance with procedures evaluated and approved by IACUC of IPB PRC Indonesia.

Figure 1: Tupaia javanicaHorsfield, 1822 Photograph by Walberto Sinaga, IPB PRC Indonesia

The hepatocytes were successfully isolated and cultured using the modified methods of Glebe *et al.*[10]. As described in their study, Glebe *et al.* [10] used a pump device to perform perfusion ofliver organ. In this study, perfusion was conducted manually with a syringe containing perfusion solution. The use of pump device in Glebe *et al*. method made it possible to control the speed and pressure of the fluid as to minimize the cell defect due to high pressure, which can happen if perfusion is done manually, as we did in this study. However, in this research the PTH culture was successfully maintained for 14 days. The morphology of the cells in this study shows uniformity as compared to that of published by Glebe *et al.* [10] which is a polygonal shape. Figure 2 shows the stages of hepatocytes cell proliferation on day one, day four, day seven and day fourteen. On day one, the cells were seen elongated-shape and arranged as individuals, whereas on day four the colony started to form and the cells were in polygonal shapes, and on day fourteen the cells reached 80% confluence .The polygonal-shape of the PTHs showed in this study was similar to the morphology of the hepatocytes from the study published by Walter *et al*. [6].

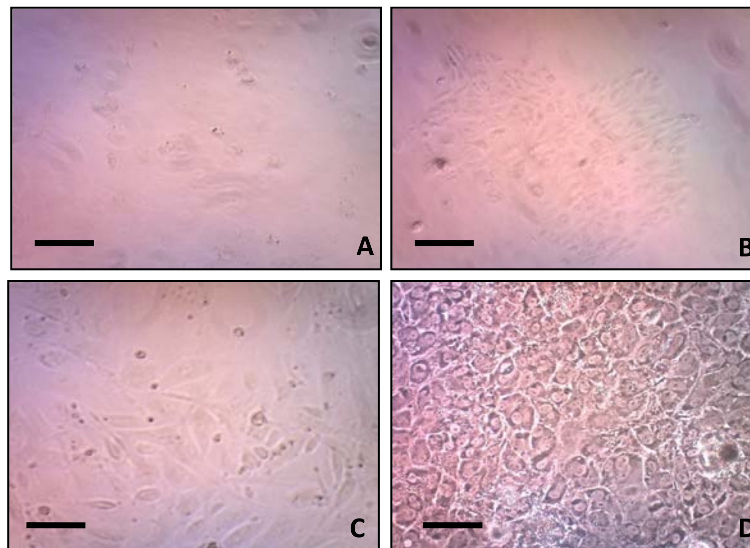

Figure 2: Primary *Tupaia* hepatocytes (PTH). (A) day one, (B) day four, (C) day seven; (D) day fourteen (Bar = 200 µm)

Transformation was performed on day fifteen and the cells were observed everyday. The morphology of primary culture at transformation stage on day one, seven, twenty one and twenty eight did not show any changes (Figure 3).

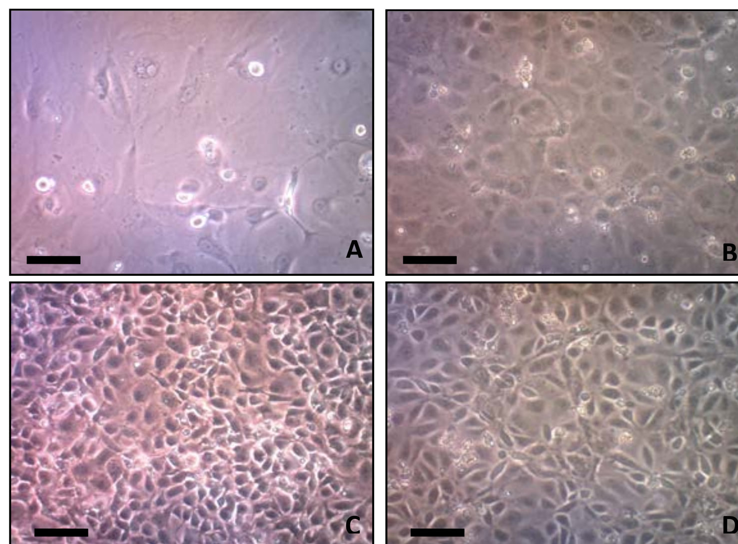

Figure 3: Transformed PTH. (A) day one, (B) day seven, (C) day twenty one; (D) day twenty eight (Bar = 200 µm)

Figure 4 shows telomerase concentration in a transformed PTH. Sample 1 to 5 are the primary cultures that have been transformed by Lenti-SV40 and harvested on day eight (no 1), day 22 (no 2), day 29 (no 3), day 50 (no 4) and day 64 (no 5). Sample no 6 is PTH, sample no 7 is immortal culture cell of Chang (ATCC CCL-13) which was taken from liver and sample no 8 is PBMC (*peripheral blood mononuclear cell*).

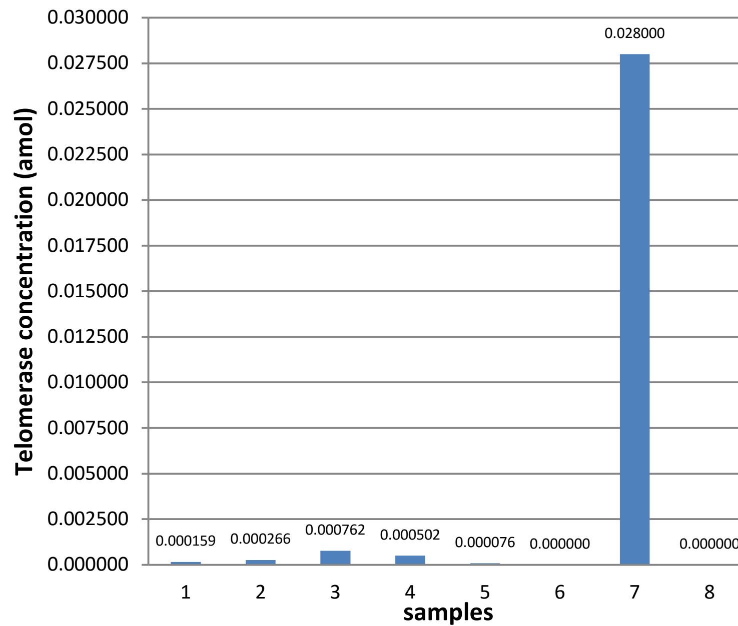

Figure 4: Telomerase concentration (amol) in transformed PTH

Telomere is a DNA segment found at the end of eukaryote cell chromosome. The main function of telomere is to protect DNA from deterioration and to maintain the stability of the chromosome. Any changes in telomere are connected to aging process and carcinogenesis. The telomere's intactness is maintained by telomerase enzyme. The aging process is associated with the shortening of telomere. In human, the length of telomere decreases proportionally with age. Fetus cell or tissue contains longer telomere compared to adult somatic cell. Telomerase enzyme have significant role in maintaining cancer cell proliferation. The activity of telomerase enzyme in cancer or tumor cell is somewhat high, resulting in making cancer cell immortal and perform continuous proliferation. The length of the telomere is shorter than that of normal cell or tissue [12].

In PTH and PBMC, telomerase enzyme activity was not apparent. This finding confirms the above literature that states that telomerase in normal cell, enzyme activity decreases in conjunction with the age or is very low, enabling the two types of cultures the negative control. Chang culture cell shows a very high telomerase activity; this is because Chang cell is an immortal cell from a liver cancer. The enzyme is generally expressed in human cancer cell. The increase of telomerase expression results in vulnerability against cancer cell; this matter differentiate cancer cell and normal cell in the body, despite enzyme activity being detected in normal cell [13].

In transformed PTH, telomerase activity was detected on day 8 (0.000159 amol). Concentration of telomerase increased and the highest concentration was on day 29 (0.000762 amol); after that, the telomerase concentration decreased on day 50 (0,000502 amol) and day 64 (0,000076 amol). Compared with telomerase concentration of Chang cell culture (0.028 amol), showed high concentration difference. Chang cell culture was immortal cell culture whereas in this study the culture was on immortal process, that it resulted a small amount of telomerase.

The result of PCR of S HBV gene (456 bp) from the infected culture cell supernatant showed the existence of

DNA amplification from day one to day eight (Figure 5). The figure performed the thickness of DNA band which showed GiHBV replication with various concentration.

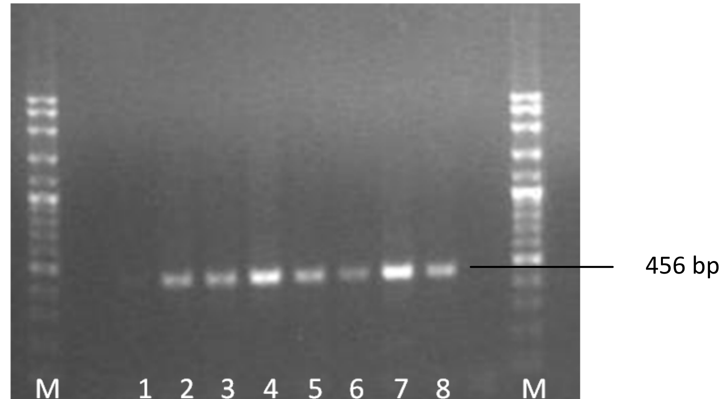

Figure 5: Electrophoresis result of DNA GiHBV infected in transformed PTH (M= marker; 1-8=supernatant day 1 to 8)

This finding was confirmed by virus titer that was detected up to day eight using real time PCR method (Figure 6). The highest viral copy number was on day 4 (158,000 viral copy number), and decreased until day 8. Both findings showed that the transformed PTH was capable of supporting virus replication up to day eight post inoculation.

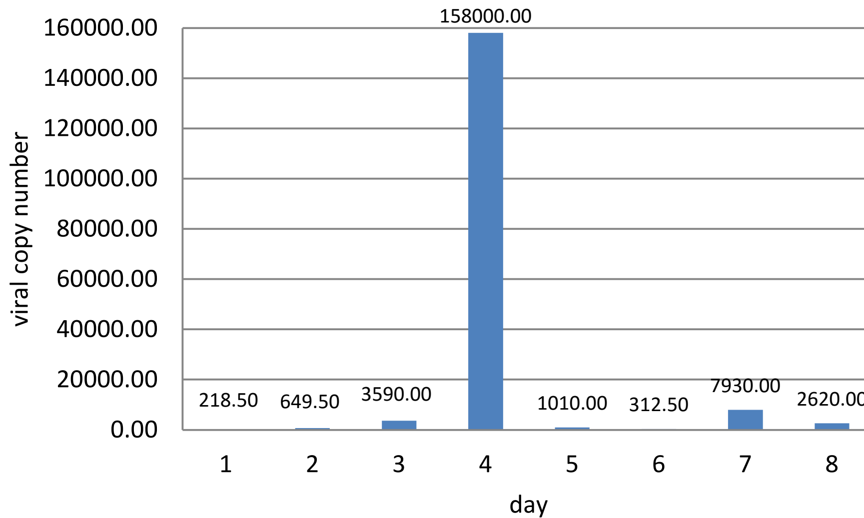

Figure 6: GiHBV replication on transformed PTH

4. Conclusion

The PTH was successfully developed for fourteen days to reach 80% confluent and then transformed. The cell morphology of PTH and the transformed PTH did not show any changes. The culture in this transformation stage was able to replicate GiHBV up to day eight post inoculation. Further study will be required to extend

culture cultivation period that immortal culture can be obtained. One major obstacle of this study was that all animals used in the study were obtained from the wild; although these animals had undergone quarantine and conditioning protocol before the study began, their overall conditions were still relatively unchanged afterward. One solution to this problem is to establish breeding of *Tupaia* so the required criteria of the animals can be more uniform.

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